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Arabidopsis RNA Polymerases IV and V Are Required To Establish H3K9 Methylation, but Not Cytosine Methylation, on Geminivirus Chromatin

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ABSTRACT

In plants, RNA-directed DNA methylation (RdDM) employs small RNAs to target enzymes that methylate cytosine residues. Cytosine methylation and dimethylation of histone 3 lysine 9 (H3K9me2) are often linked. Together they condition an epigenetic defense that results in chromatin compaction and transcriptional silencing of transposons and viral chromatin. Canonical RdDM (Pol IV-RdDM), involving RNA polymerases IV and V (Pol IV and Pol V), was believed to be necessary to establish cytosine methylation, which in turn could recruit H3K9 methyltransferases. However, recent studies have revealed that a pathway involving Pol II and RNA-dependent RNA polymerase 6 (RDR6) (RDR6-RdDM) is likely responsible for establishing cytosine methylation at naive loci, while Pol IV-RdDM acts to reinforce and maintain it. We used the geminivirus *Beet curly top virus* (BCTV) as a model to examine the roles of Pol IV and Pol V in establishing repressive viral chromatin methylation. As geminivirus chromatin is formed *de novo* in infected cells, these viruses are unique models for processes involved in the establishment of epigenetic marks. We confirm that Pol IV and Pol V are not needed to establish viral DNA methylation but are essential for its amplification. Remarkably, however, both Pol IV and Pol V are required for deposition of H3K9me2 on viral chromatin. Our findings suggest that cytosine methylation alone is not sufficient to trigger *de novo* deposition of H3K9me2 and further that Pol IV-RdDM is responsible for recruiting H3K9 methyltransferases to viral chromatin.

IMPORTANCE

In plants, RNA-directed DNA methylation (RdDM) uses small RNAs to target cytosine methylation, which is often linked to H3K9me2. These epigenetic marks silence transposable elements and DNA virus genomes, but how they are established is not well understood. Canonical RdDM, involving Pol IV and Pol V, was thought to establish cytosine methylation that in turn could recruit H3K9 methyltransferases, but recent studies compel a reevaluation of this view. We used BCTV to investigate the roles of Pol IV and Pol V in chromatin methylation. We found that both are needed to amplify, but not to establish, DNA methylation. However, both are required for deposition of H3K9me2. Our findings suggest that cytosine methylation is not sufficient to recruit H3K9 methyltransferases to naive viral chromatin and further that Pol IV-RdDM is responsible.

Repressive chromatin methylation suppresses the expression of transposable elements and DNA viruses and leads to the establishment of transcriptional gene silencing (TGS). Plants employ RNA-directed DNA methylation (RdDM) to target methylation of cytosine residues and use cytosine methylation and associated histone 3 lysine 9 dimethylation (H3K9me2) to silence invasive DNAs, such as transposons and geminiviruses. As this study addresses the roles of RNA polymerases in repressive methylation of geminivirus chromatin, an overview of relevant pathways and their interrelationships is presented (1, 2).

In the reference plant *Arabidopsis thaliana*, the canonical RdDM pathway (also known as Pol IV-RdDM) involves plant-specific DNA-dependent RNA polymerases IV and V (Pol IV and Pol V) (3–6). These multisubunit enzymes are related to Pol II and are capable of transcribing methylated DNA (7–9). Pol IV, likely in collaboration with the putative chromatin remodeling factor CLASSY 1 (CLSY1) (10), acts upstream in RdDM to create noncoding (nc) transcripts that are substrates for RNA-dependent RNA polymerase 2 (RDR2). The resulting double-stranded RNA (dsRNA) subsequently is processed into 24-nucleotide (nt) small interfering RNAs (siRNAs) by DICER-LIKE 3 (DCL3). The siRNA guide strand is then incorporated into an ARGONAUTE 4 (AGO4)- or AGO6-containing RNA-induced silencing complex (RISC) and targets RISC by base pairing with Pol V scaffold transcripts that remain associated with template DNA. The tethered RISC recruits DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2) to carry out *de novo* cytosine methylation in symmetric (CG and CHG, where H is A, T, or C) and asymmetric (CHH) contexts.

Once established, symmetric methylation is perpetuated through DNA synthesis by RdDM-independent mechanisms involving METHYLTRANSFERASE 1 (MET1), a homologue of mammalian DNMT1, and CHROMOMETHYLASE 3 (CMT3). Cytosine

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methylation is strongly associated with repressive histone methylation (H3K9me2) carried out by the SU(var)3-9 homologue KRYPTONITE (KYP; also known as SUVH4), SUVH5, and SUVH6. While CG methylation maintenance by MET1 is independent of histone methylation, CHG maintenance employs CMT3 and KYP in a self-reinforcing loop (11). The chromodomain of CMT3 binds H3K9me2, while KYP is targeted by SET- and RING-associated (SRA) domains that bind methylated DNA (12–14). In contrast, maintenance of asymmetric CHH methylation in euchromatin requires continuous Pol IV-RdDM acting through a self-reinforcing loop involving SAWADEE HOMEODOMAIN HOMOLOG 1 (SHH1) and SUVH2/9, which recruit Pol IV and Pol V, respectively. SHH1 is a dual-specificity chromatin reader that recognizes H3K9me2 and unmethylated H3K4 (15, 16), while SUVH2 and SUVH9 are histone methyltransferase-like proteins that lack catalytic activity but bind methylated DNA (17, 18). In the heterochromatin that contains linker histone H1, CHH methylation maintenance becomes RdDM independent and is accomplished by CMT2, which, like CMT3, binds H3K9me2 (19, 20).

While Pol IV-RdDM accounts for maintenance of asymmetric cytosine methylation, recent studies have revealed an alternative pathway for *de novo* methylation of naive sequences, including active transposable elements (TEs), transacting siRNA (tasiRNA) loci, and an active *FLOWERING WAGENINGEN* (*FWA*) epiallele (21–26). Termed RDR6-RdDM, this pathway involves components previously associated with posttranscriptional gene silencing (PTGS). In this scenario, dsRNA generated by Pol II and RDR6 is processed by DCL2/DCL4 into 21- to 22-nt siRNAs that guide AGO4 or AGO6 to Pol V scaffold transcripts. Pol II, or consequent cytosine methylation, could recruit Pol IV and Pol V, triggering Pol IV-RDR2-DCL3 production of 24-nt siRNAs and activating Pol IV-RdDM at the affected locus. Alternatively or additionally, high levels of Pol II-RDR6-generated dsRNA could saturate DCL2/DCL4 and allow processing by DCL3 (27). Although mechanistic details remain to be elucidated and may vary between different types of sequences, these studies commonly suggest that Pol IV-RdDM does not initiate DNA methylation at naive loci but primarily reinforces and amplifies preexisting cytosine methylation.

Clearly, much has been learned about pathways that establish and reinforce cytosine methylation and of links between cytosine and H3K9 methylation that are responsible for maintaining these epigenetic marks. However, gaps remain in our understanding of the establishment of H3K9 methylation, and potential roles for Pol IV and Pol V in this process have not been rigorously examined.

Geminiviruses encapsidate small (2.5- to 3.0-kb) circular single-stranded DNA (ssDNA) genomes that replicate in the nuclei of infected cells through dsDNA intermediates that associate with histones to form minichromosomes. Viral chromatin acts as a template for replication and transcription, both of which are almost entirely dependent on host machinery (28, 29). Plants have formidable defensive arsenals, including RNA-based defenses, which must be neutralized to ensure pathogen success (30, 31). Two branches of RNA silencing target geminiviruses (32, 33). Viral mRNAs are subject to PTGS (34, 35), while methylation-mediated TGS targets viral minichromosomes (36, 37). As a counter-defense, geminiviruses and associated satellite DNAs encode pathogenicity factors that employ multiple mechanisms to suppress both PTGS and TGS and inhibit repressive methylation (38–47). The hypersensitivity of methylation-deficient mutant plants to geminivirus infection and the existence of numerous viral countermeasures underscore the defensive value of viral chromatin methylation. Moreover, because viral chromatin must be formed and modified *de novo* in infected cells, geminiviruses provide unique systems for examining processes that target and establish epigenetic modifications.

In the studies presented here, the geminivirus *Beet curly top virus* (BCTV) was used to assess the roles of Pol IV and Pol V in the establishment of viral DNA and H3K9 methylation. We found that while Pol IV and Pol V are required to amplify cytosine methylation, they are not necessary for its establishment or for the production of virus-derived siRNAs, including the 24-nt size class associated with Pol IV-RdDM. However, both Pol IV and Pol V are required for deposition of H3K9me2 on viral chromatin.

MATERIALS AND METHODS

***Arabidopsis* mutants.** Mutants were obtained from the Arabidopsis Biological Resource Center at The Ohio State University. The following seed stocks were used: wild-type Col-0 (CS60000) Columbia ecotype, *clsy1* (SALK_018319/At3g42670), *nrpd1* (SALK_128428/CS66150 and CS66151/At1g63020) (4), *nrpe1* (*nrpd1b-11*; SALK_022919/At2g40030), and *nrpd/e2* (*nrpd2a*; SALK_109513C, SALK_090385C, and SALK_095689/At3g23780). Similar phenotypes were observed, and representative results are shown when multiple mutant lines were tested. All mutants were homozygous, as verified by genotyping. Plants were reared in growth rooms at 22°C with a 16-h light/8-h dark cycle.

Virus inoculation. *Arabidopsis* plants were agroinoculated with BCTV or *Cabbage leaf curl virus* (CaLCuV) as previously described (36, 48). At least 32 plants, inoculated within 5 days of bolting, were used in each experiment. For each sample, tissue was pooled from at least three infected plants. For recovery experiments with BCTV L2$^-$, infected primary inflorescence tissue was harvested after ~21 days, and plants were allowed to continue growing under the same conditions. Secondary inflorescence tissue was harvested 14 to 21 days following the primary harvest. Similar results were obtained with two different mutants (BCTV L2-1 and L2-2), both of which have been described (49). Thus, BCTV L2$^-$ is used throughout to indicate either mutant. Tissue was harvested from CaLCuV-infected plants 14 days postinoculation.

Bisulfite sequencing. Bisulfite sequencing was performed as described previously (36, 50). As an internal control, a bacterial plasmid containing CaLCuV DNA was added to BCTV-infected sample extracts prior to treatment with bisulfite reagent. Alternatively, as a parallel control, a plasmid containing BCTV DNA was added to an uninfected plant DNA extract. Data were accepted only if control DNA conversion was complete (12 clones analyzed per treatment). In some cases, plasmid DNA was methylated *in vitro* with CG methylase before being added to plant extracts, and data were accepted only when conversion was complete at non-CG sites and all CG sites remained unconverted. The forward and reverse primers used to amplify the BCTV intergenic region (IR) and coat protein (CP) coding regions following bisulfite conversion are listed in Table 1. Data were analyzed and dot plots prepared using Kismeth (51).

ChIP. Chromatin immunoprecipitation (ChIP) was performed as described previously (36, 52). Symptomatic *Arabidopsis* inflorescence tissue (0.3 g) was harvested from a pool of at least 3 plants. Immunoprecipitation was carried out overnight at 4°C using the commercially available antibody αFLAG (F1804; Sigma), αH3K9me2 (ab1220; Abcam), or αH3-Ac (acetylated at lysine 14 and 9) (06-599; Millipore). The αGAL4 DNA binding domain (sc-577; Santa-Cruz) was used as a nonspecific IgG control. Precipitated DNA (1 μl) was used as the PCR template with primers specific for viral DNA or a control sequence (Table 1). Semiquantitative PCR (sqPCR) was performed with an annealing temperature of

TABLE 1 Oligonucleotides used in this study[a]

No.	Designation	Sequence (5′ to 3′)	Assay
1	BCTV147CR	TCTATTAACCAAACAACAAATCT	Bisulfite
2	BCTV2640CF	GGGATATGTAAGAAATATG	Bisulfite
3	bisBCTV1CP2F	ATGAGGAAATATAAAAGAAATAAGTATAAAATGT	Bisulfite
4	bisBCTV362CP2R	ACCCAACATCCTTTTCTATTATAATC	ChIP (sqPCR and qPCR)
5	qAct2FA118	GAGAGATTCAGATGCCCAGAAGTC	ChIP (sqPCR and qPCR)
6	qAct2RA119	TGGATTCCAGCAGCTTCCA	ChIP (sqPCR and qPCR)
7	qIGN5FA193	AAGCCCAAACCATACACTAATAATCTAAT	ChIP (sqPCR and qPCR)
8	qIGN5RA194	CCGAATAACAGCAAGTCCTTTTAATA	ChIP (sqPCR and qPCR)
9	BCTVIRqRTF	ATTGGACTCCGATGACGTGGCTTA	ChIP (sqPCR and qPCR)
10	BCTVIRqRTR	ACGGATGGCCCTTATGAGAGTTGT	ChIP (sqPCR and qPCR)
11	BCTVCPF1122	TTCCCGATAACGGTCAGGCTAT	ChIP (sqPCR)
12	BCTVCPR1273	TGGCATTGAAGGAGCCTTGT	ChIP (sqPCR)
13	BCTVCP2F	ACCAATGGAGGAGACCTGTGACAA	ChIP (qPCR)
14	BCTVCP2R	ATCCTATACCACCAGCACCCAACA	ChIP (qPCR)
15	BCTVCPF57	TCAAAGTGCGTGGCCGAAGAA	21-nt marker
16	BCTVIRsR-2	TCTTCAGGAAGTTTCCCGCTCA	22-nt marker
17	BCTVIRqRTR	ACGGATGGCCTTATGAGAGTTGT	24-nt marker
18	BCTVIRsR-2	TGAGCGGGAAACTTCCTGAAGA	IR probe
19	BCTVIRsF-1	GACACGTGGAAGGGTACTGTAGAA	IR probe
20	BCTVIRsF-2	TAAGATTTGTTGACTGGTCAATAGAAGG	IR probe
21	BCTVIRsR-1	GTTTTGATTGGTTGCCCATTTTTTCG	IR probe
22	BCTVCPF57	TCAAAGTGCGTGGCCGAAGAA	CP probe
23	BCTVCPF1122	TTCCCGATAACGGTCAGGCTAT	CP probe
24	BCTVCPF1273	TGGCATTGAAGGAGCCTTGT	CP probe
25	qCaLCuVAIR95R	ATAGGTTTTAGAGAGAGCTCTCCTGGCGTC	ChIP (qPCR)
26	qCaLCuVAIR2579F	CGGGCTGCTAAACGAAACGATTTAGGGTTTC	ChIP (qPCR)
27	Athils6ALTRqPCRF	TTGCTTCTGCAACCTGTAATTCG	ChIP (qPCR)
28	Athils6ALTRqPCRR	GACAACATGCTCAAAGACATAAACG	ChIP (qPCR)

[a] Oligonucleotides 1 to 4 were primers for PCR amplification and cloning of the BCTV intergenic region (IR) or coat protein (CP) coding region for bisulfite sequencing. Oligonucleotides 5 to 8 were used to amplify Actin and IGN5 control regions for ChIP-sqPCR and ChIP-qPCR (56). Oligonucleotides 9 and 10 were used to amplify the BCTV IR for sqPCR and qPCR amplification after ChIP. Oligonucleotides 11 and 12 were used to amplify the BCTV CP CDS in ChIP-sqPCR, while 13 and 14 were used in ChIP-qPCR for the BCTV CP CDS. Oligonucleotides 15 to 17 served as size markers for small RNAs, while oligonucleotides 18 to 24 were end labeled with ^{32}P for use as hybridization probes to detect either BCTV IR- or BCTV CP-specific small RNAs on gel blots. Oligonucleotides 25 and 26 were used in ChIP-qPCR for the *Cabbage leaf curl virus* (CaLCuV) IR. Oligonucleotides 27 and 28 were used in ChIP-qPCR to amplify the Athila6A LTR.

55°C for all primer sets. Different cycle numbers were used depending on the primer set: 21 cycles for the BCTV IR and 26 for BCTV CP in primary infected tissue, 23 cycles for the BCTV IR and 28 for the BCTV CP in secondary infected tissue, and 35 cycles for IGN5, Actin, and Ta3. PCR products were diluted 2-fold and analyzed by gel electrophoresis. Quantitative PCR (qPCR) was performed with SYBR Supermix (170-8880; Bio-Rad), the aforementioned primer sets plus primers for the CaLCuV IR and Athila 6A, and the following cycle conditions on a Bio-Rad CFX96 unit: 95°C for 3 min, followed by cycles of 95°C for 15 s, 55°C for 30 s, and 72°C for 30 s. Cycle threshold values were converted to quantitative values using a dilution standard (containing the target of interest) measured by qPCR, normalized to individual input values, and the IgG background was subtracted.

Small RNA analysis. Small RNAs were analyzed as described previously (53), beginning with ~0.2 g of symptomatic floral tissue harvested from a pool of 3 to 4 plants. Oligonucleotides used as size markers are listed in Table 1, as are sense and antisense IR and CP coding sequence (CDS) oligonucleotides that were used as hybridization probes. rRNA loads and small RNA levels were measured using Quantity One software. Briefly, signals were measured using the Volume Analysis tool (the same area measurement was used to measure each sample). This measurement reported the sum of the intensities of the pixels inside the area outlined. Each small RNA measurement was then divided by the respective RNA load measurement. The normalized small RNA measurements were then compared to those of the wild type and are displayed as a ratio change, with Col-0 set to 1.

RESULTS

Pol IV and Pol V individually contribute to defense against geminiviruses. We previously showed that *Arabidopsis* plants deficient for Pol IV-RdDM components, cytosine and H3K9 methyltransferases, and methyl cycle enzymes are hypersusceptible to geminivirus infection (36, 54). Because a *pol IV/V* double mutant (*nrpd/e2*) was used in earlier studies, we asked whether these polymerases individually participate in antiviral defense. The Pol IV-associated, SNF2-like chromatin remodeling enzyme CLASSY 1 (CLSY1) was also included in these experiments (10). *Arabidopsis* plants homozygous for inactivating mutations in *pol IV* (*nrpd1*), *pol V* (*nrpe1*), *pol IV/V* (*nrpd/e2*), and *clsy1* were inoculated with BCTV. The *pol IV* and *pol V* mutants lack the largest catalytic subunit of each enzyme, while the *pol IV/V* double mutant is deficient for the shared second largest subunit.

All of the infected mutant plants proved hypersusceptible to BCTV compared to wild-type plants (Fig. 1A, top row). Disease ratings ranged from 3 to 4 on a subjective scale where 1 indicates symptoms typically observed with BCTV-infected wild-type plants and 4 indicates very severe leaf curling, deformation of floral tissues, and stunting. These results clearly indicate that Pol IV and Pol V independently contribute to antiviral defense and are consistent with functional association of CLSY1 and Pol IV.

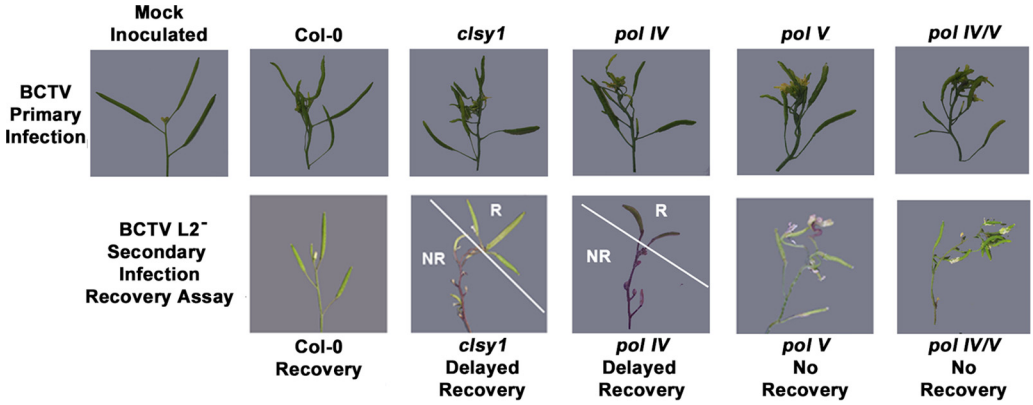

FIG 1 Plants deficient for Pol IV, Pol V, and CLSY1 are hypersusceptible to geminivirus infection. Photographs taken under a dissecting microscope show floral heads from infected wild-type (Col-0), *clsy1*, *pol IV*, *pol V*, and *pol IV/V* plants. Primary shoots infected with BCTV (top row) were photographed ~21 days postinoculation, and photographs of secondary infected shoots infected with BCTV L2$^-$ (bottom row) were taken ~14 days after harvest of primary tissue (~35 days postinoculation). Photographs are representative of 32 plants per treatment. Note increased floral deformation in mutant compared to wild-type plants during primary infection. During secondary infection (recovery assay), the absence of symptoms indicates recovery in wild-type plants, while *pol V* and *pol IV/V* mutants do not recover and show severe symptoms. The *pol IV* and *clsy1* mutants exhibit delayed recovery. Nonrecovered (NR) and recovered (R) tissue was harvested as indicated (white lines).

Pol IV and Pol V amplify viral DNA methylation but are not required for its establishment. We have found analysis of host recovery from geminivirus infection to be a sensitive method for the identification and study of methylation pathway components and took this approach to further examine Pol IV and Pol V. BCTV encodes L2 protein (also known as C2), a pathogenicity factor known to suppress cytosine methylation and TGS (40). *Nicotiana benthamiana* and *Arabidopsis* plants inoculated with L2-deficient BCTV (BCTV L2$^-$) exhibit recovery, where shoots arising after the establishment of a systemic infection show symptom remission and contain only small amounts of viral DNA (36, 49). In our recovery experiments, infected shoots (primary infected tissue) are harvested after ~21 days, and new shoots (secondary infected tissue) are allowed to develop from axillary meristems. Secondary shoots of wild-type plants rarely recover from BCTV but invariably recover from BCTV L2$^-$. Viral DNA in recovered tissue is hypermethylated in the intergenic region (IR), an ~300-bp master control region that contains divergent promoters flanking the origin of replication (Fig. 2D). In contrast, *ago4*, *dcl3*, and *drb3* mutants cannot recover from BCTV L2$^-$ and fail to hypermethylate the IR, indicating that the Pol IV-RdDM pathway is required for host recovery (36, 54).

Following infection of wild-type plants with BCTV L2$^-$, all secondary shoots showed symptom remission (recovery) as expected, but *pol V* and *pol IV/V* mutants failed to recover as secondary shoots continued to show severe symptoms (Fig. 1A, bottom row). Interestingly, *pol IV* and *clsy1* mutants displayed a novel, delayed recovery phenotype: secondary shoots at first displayed severe disease symptoms but over time showed symptom remission, suggesting the presence of functionally redundant activities for both enzymes. The strikingly similar phenotypes are most consistent with Pol IV and CLSY1 acting together in the methylation pathway.

Previous studies have shown that cytosines in the IR of individual BCTV genomes are either mostly methylated in all sequence contexts or mostly unmethylated. This reflects equilibrium between active and repressed viral genomes in infected plants, which is shifted toward repressed (methylated) genomes during recovery (36, 54). The region of the BCTV IR analyzed contains 42 cytosines in the following contexts: 10 CG, 7 CHG, and 25 CHH (where H is any nucleotide except G). Bisulfite sequencing of viral DNA obtained from secondary recovered tissue of wild-type plants confirmed that the IR was hypermethylated (~60% of total cytosine residues methylated) (Fig. 2A and B), and the high density of asymmetric CHH methylation observed is a signature of *de novo* methylation (23). Remarkably, similar hypermethylation (>60% of cytosines methylated) was evident in the recovered portions of shoots from *pol IV* and *clsy1* mutants but not in nonrecovered portions of the same shoots (~40 to 45% of cytosines methylated), confirming that hypermethylation is characteristic of recovered tissue. The nonrecovered *pol V* plants also failed to hypermethylate the IR (~40% cytosines methylated), while the nonrecovered *pol IV/V* plants displayed a somewhat higher level of methylation (~50% of cytosines methylated; see Discussion). In short, nonrecovered *pol IV* and *pol V* plants are able to methylate a substantial fraction of cytosines in the BCTV L2$^-$ IR but cannot achieve the hypermethylation observed in wild-type plants. This allows us to conclude that Pol IV and Pol V are not required to establish cytosine methylation but are necessary for amplification.

To verify the significance of our results, we combined bisulfite sequencing data sets from these and previous experiments with different mutants, all of which were performed under similar conditions with the same IR primer set (54) (Fig. 2C). Clones from secondary tissues of BCTV L2$^-$-infected plants classified as recovered (Col-0 background, $n = 93$) were grouped together. This group included clones from wild-type plants, *dcl4* and *drb4* mutants, and the recovered portions of *pol IV* and *clsy1* shoots. Mean total cytosine methylation was found to be ~64%. The analysis was repeated for all bisulfite clones from nonrecovered tissues (Col-0 background, $n = 90$). Included in the nonrecovered group were clones from *dcl3*, *drb3*, and *pol V* plants and the nonrecovered portions of *pol IV* and *clsy1* shoots. Because *pol IV/V* double mutants might retain some residual function, they were not included in this analysis (see Discussion). Mean total cytosine methylation in nonrecovered mutant plants was ~42%. Statistical

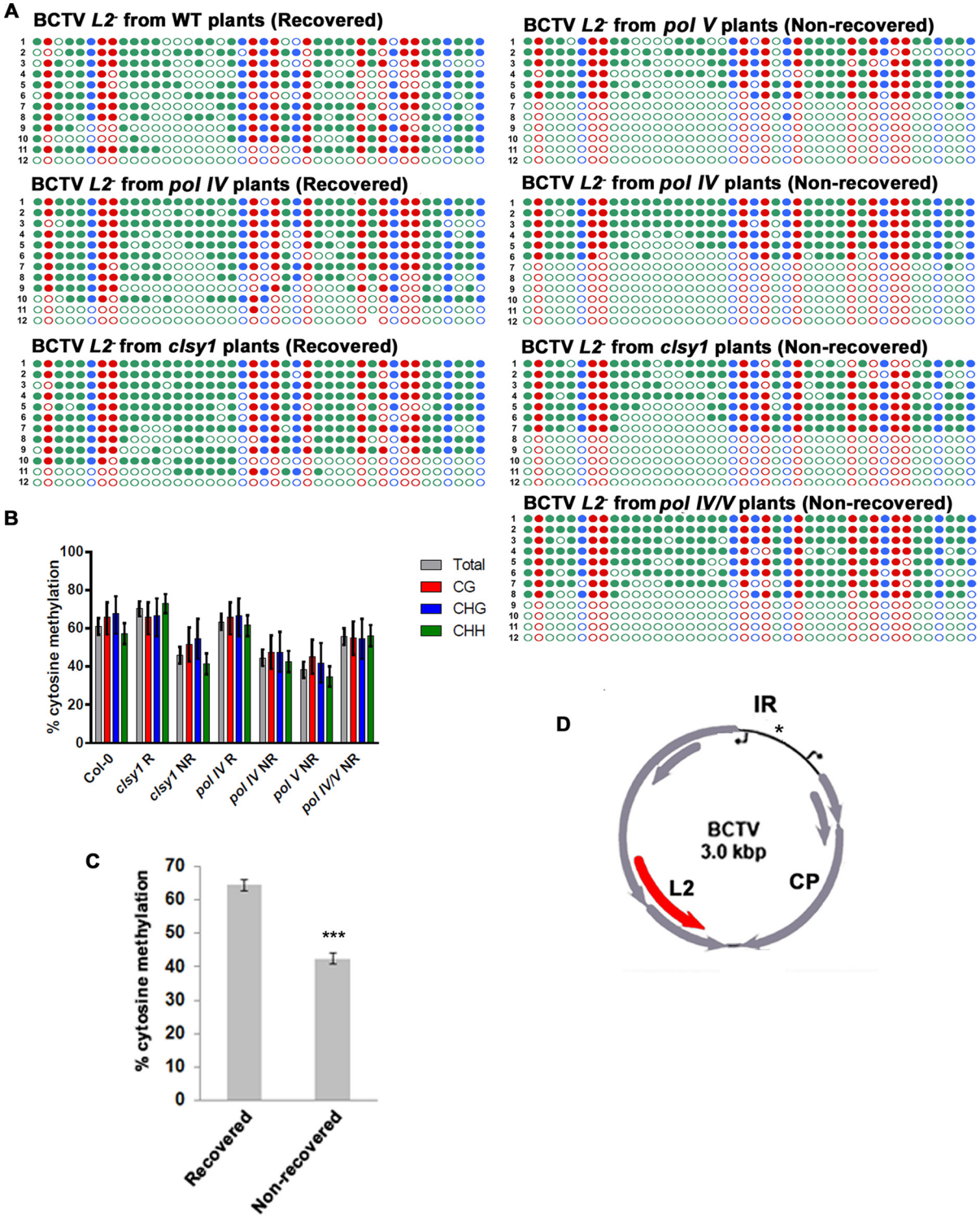

FIG 2 Pol IV and Pol V are not required to establish viral DNA methylation. (A) Methylation status of the BCTV L2$^-$ IR from secondary tissue of infected wild-type and mutant plants. Samples from recovered (R) and nonrecovered (NR) regions of shoots from *clsy1* and *pol IV* mutants were separately analyzed (Fig. 1). Samples consisted of tissue pooled from three plants. DNA was treated with bisulfite, and PCR was performed to amplify the viral strand. PCR products were cloned and sequenced. Rows depict individual clones (12 per treatment) organized from most to least methylated. Each circle represents a cytosine in the IR (42 total) with CG (10) in red, CHG (7) in blue, and CHH (25) in green. Filled circles indicate a methylated cytosine. (B) Histograms show the percentage of methylated cytosine residues in different sequence contexts. Total methylation is indicated in gray. Bars indicate Wilson score interval 95% confidence limits. (C) Compilation of bisulfite data from wild-type and mutant plants. Clones from secondary tissues of BCTV L2$^-$-infected plants classified as recovered ($n = 93$) or nonrecovered ($n = 90$) were placed in separate groups (see the text). Asterisks indicate mean cytosine methylation levels are significantly different ($P < 0.001$), as determined by Student's *t* test. (D) The diagram depicts the dsDNA replicative form of BCTV. Genes are shown as gray arrows, with the L2 silencing suppressor in red. The replication initiation site within the IR is marked with an asterisk, and Pol II transcription starts are indicated by right-angle arrows.

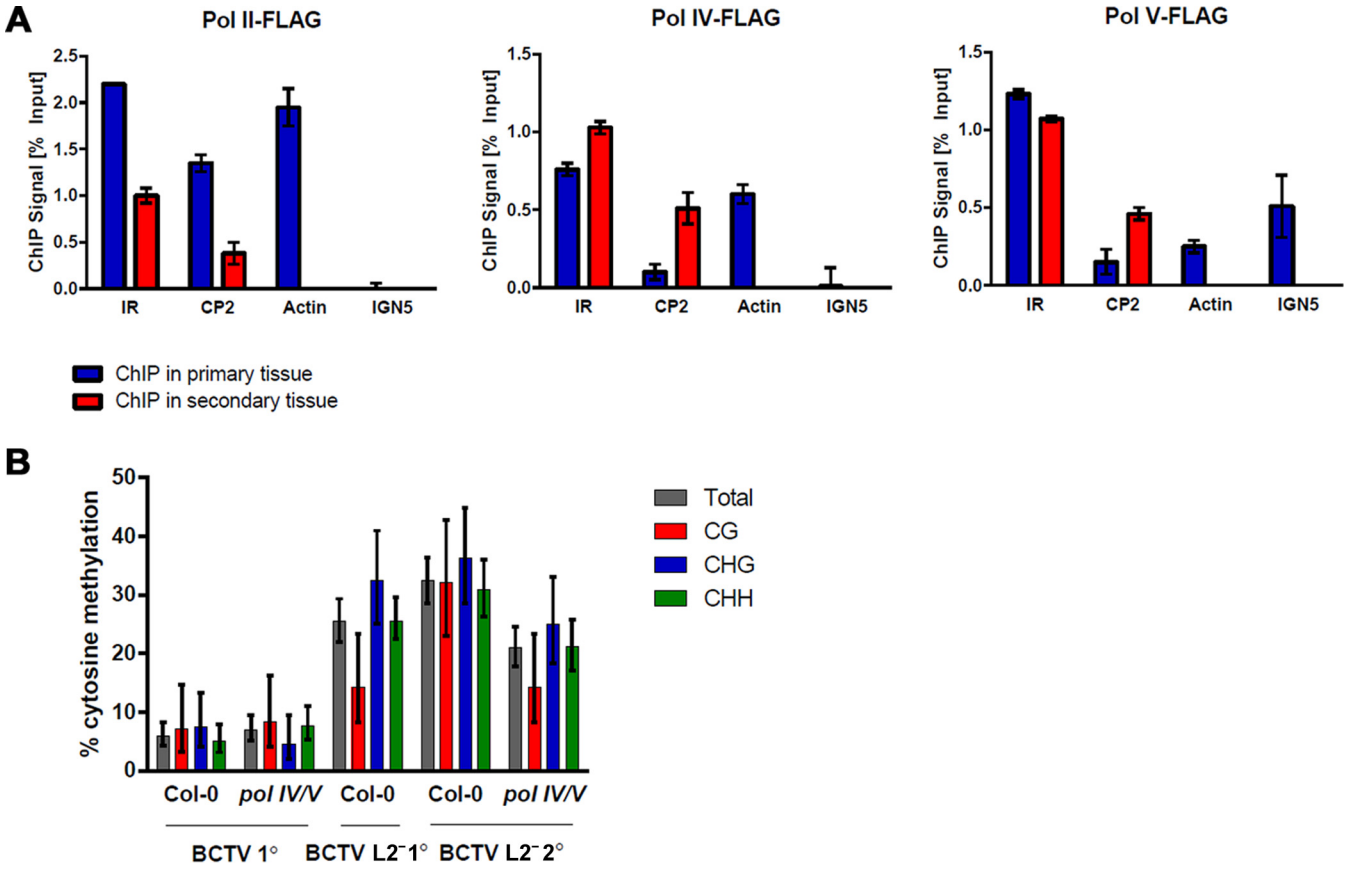

FIG 3 Pol II, Pol IV, and Pol V associate with the BCTV genome. (A) Representative ChIP experiments with extracts from infected, transgenic plants expressing FLAG-Pol II, Pol IV, or Pol V subunits are shown. Extracts were obtained from BCTV-infected primary tissue (blue bars) and BCTV L2$^-$-infected secondary tissue (red bars), and in all cases samples consisted of pooled tissue from at least three plants. DNA precipitated with FLAG antibody was analyzed using qPCR performed in triplicate with at least three independent samples (three biological replicates with three technical replicates each). Primer sets to amplify the BCTV IR and the CP CDS (CP2) were employed, as were control primers for actin and IGN5. IgG was a background control. The graphs depict signal as a percentage of input. Bars indicate standard errors. (B) Methylation of the CP CDS in nonrecovered and recovered tissues. Cytosine methylation in the CP CDS was assessed in primary (1°) tissue from wild-type and *pol IV/V* plants infected with BCTV or BCTV L2$^-$ and in BCTV L2$^-$-infected secondary (2°) tissue of recovered wild-type plants and nonrecovered *pol IV/V* plants. Infected inflorescence tissue was harvested from a pool of at least three plants, and DNA was isolated and treated with sodium bisulfite to convert unmethylated cytosines to uracil. PCR with primers designed to amplify CP2 (Table 1) was performed, and products were cloned and sequenced. The region examined contains 46 cytosines: 7 CG, 11 CHG, and 28 CHH. Twelve clones were examined per treatment. Histograms show total cytosine methylation levels (gray bars) as well as methylation in different sequence contexts (CG in red, CNG in blue, and CHH in green). Bars represent Wilson score interval 95% confidence limits.

analysis verified that the difference in BCTV IR cytosine methylation levels between recovered and nonrecovered groups is highly significant ($P < 0.001$), with minimal variation within groups.

The results of these experiments reveal that Pol V is essential for host recovery from geminivirus infection, while Pol IV and CLSY1 play important roles. They also clearly demonstrate that Pol IV and Pol V are not required to establish *de novo* cytosine methylation on the BCTV genome but are needed to amplify methylation.

Pol IV and Pol V physically associate with viral DNA. Our genetic studies suggest that Pol IV and Pol V associate with viral DNA. To confirm this, ChIP experiments were carried out using transgenic *Arabidopsis* lines expressing the largest FLAG epitope-tagged Pol IV or Pol V subunits in place of cognate wild-type polypeptides (55). A transgenic line expressing the second largest FLAG-tagged subunit of Pol II was also included in this study. Plants were inoculated with BCTV or BCTV L2$^-$, and only those in which the Pol IV and Pol V transgenes complemented the corresponding mutations (i.e., plants were not hypersusceptible and recovered from BCTV L2$^-$) were used in these experiments. ChIP was performed with FLAG antibody, and precipitated DNA was analyzed using qPCR (Fig. 3A) or sqPCR (Fig. 4). Actin was a positive control for Pol II, while IGN5 was a positive control for Pol V (56). In our hands, Pol IV reproducibly associated with the actin locus. Primers to amplify the BCTV IR and the coat protein coding sequence (CP CDS) were used to monitor polymerase association with viral DNA. CP2 primers were used for qPCR, while semiquantitative experiments employed CP1 primers (Table 1).

We found that all three polymerases associate with the IR during primary infection, when methylation is largely confined to promoter regions (41). In contrast, substantial signal was detected only for Pol II when CP CDS primers were employed (Fig. 3A and 4). In secondary recovered tissue, when the IR is hypermethylated, all three polymerases again were evident at the IR, although Pol II signal was reduced at both the IR and the CP CDS compared to primary tissue. In contrast, Pol IV and Pol V occupancy at the IR

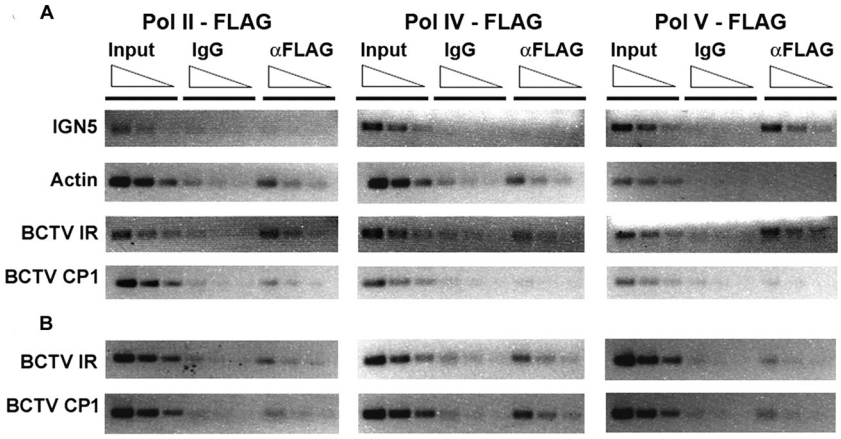

FIG 4 ChIP analysis (sqPCR) of Pol II, Pol IV, and Pol V association with the BCTV genome. Results of ChIP experiments with extracts from infected, transgenic plants expressing FLAG-Pol II, Pol IV, or Pol V subunits are shown. (A) Extracts were obtained from BCTV-infected primary tissue. (B) Extracts were obtained from BCTV L2$^-$-infected secondary tissue. Using DNA precipitated with FLAG antibody, PCR products (serial 2-fold dilutions shown) were generated with IR primers and CP1 primers or with IGN5 and actin primers (controls). In all cases samples consisted of tissue pooled from at least three plants. The experiments shown are representative of at least three independent trials. IgG was a background control.

was similar or greater than that in primary tissue and was considerably increased at the CP CDS, suggesting that these polymerases spread into coding regions during recovery.

To determine whether Pol IV and Pol V occupancy of the CP CDS correlated with increased cytosine methylation, bisulfite sequencing was performed. The region analyzed contains 46 cytosines in the following contexts: 7 CG, 11 CHG, and 28 CHH. This analysis confirmed that the BCTV CP CDS is sparsely methylated during primary infection of wild-type plants (~6% of total cytosines methylated) and methylation levels were unchanged in *pol IV/V* mutant plants, consistent with the absence of Pol IV and Pol V from this locus during primary infection (Fig. 3B). However, a considerably higher level of CP CDS methylation (~25%) was observed in BCTV L2$^-$-infected primary tissue, which moderately increased (to ~30%) in recovered, secondary tissue. In comparison, methylation observed in nonrecovered secondary tissue from BCTV L2$^-$-infected *pol IV/V* mutants was reduced to ~20%. Thus, the CP CDS is not a significant methylation target during primary infection with wild-type virus, although methylation is increased in the absence of the L2 suppressor and further increased in a Pol IV/V-dependent manner during recovery.

Taken together, these results support our genetic studies by showing that Pol IV and Pol V physically associate with the viral genome in ways that are generally consistent with phenotypes and cytosine methylation levels observed during infection and recovery.

Pol IV and Pol V contribute to the biogenesis of virus-derived siRNAs. In *Arabidopsis*, Pol IV plays a major role in 24-nt siRNA biogenesis, while Pol V is additionally required for 24-nt siRNA accumulation at some loci (57, 58). Previous studies have shown that siRNAs of all size classes (21, 22, and 24 nt) spanning the entire geminivirus genome are generated during infection, with the DCL3-dependent 24-nt class being a conspicuous component (35, 59–61). We therefore examined the roles of Pol IV and Pol V in the production of BCTV-derived siRNAs.

Total RNA was isolated from primary tissue of BCTV-infected wild-type plants and *clsy1*, *pol IV*, *pol V*, and *pol IV/V* mutants, and siRNAs were visualized by Northern blotting with probes specific for the viral IR. Although some variation was observed, *clsy1*, *pol IV*, and *pol V* mutants on average experienced only moderate reductions (~15 to 30%) in total BCTV-specific siRNAs compared to wild-type plants, and again the *pol IV/V* mutant showed the mildest phenotype (Fig. 5) (see Discussion). The relative levels of 21/22-nt and 24-nt siRNAs also were not substantially altered.

These data clearly show that Pol IV and Pol V are not required for production of virus-derived siRNAs, a result in keeping with their dispensability for establishing cytosine methylation. Thus, transcription by another polymerase, most likely Pol II, can serve as the initial source of virus-derived siRNAs of all size classes. However, consistent with their presence at the viral IR, Pol IV and Pol V contribute to siRNA biogenesis.

Pol IV and Pol V are individually required for establishment of H3K9 methylation on viral chromatin. Reflecting equilibrium between active and repressed genomes in infected plants, histone posttranslational modifications characteristic of active (histone H3 acetylated at lysine 9 and 14; H3-Ac) and repressed (H3K9me2) chromatin are present at the viral IR (36). Not surprisingly, ChIP-bisulfite experiments showed that H3K9me2 is strongly associated with highly methylated viral DNA (54). Therefore, we evaluated the roles of Pol IV and Pol V in the establishment of H3K9me2 on geminivirus chromatin.

Chromatin modifications were analyzed by ChIP using extracts from primary infected tissue harvested from BCTV- or CaLCuV-inoculated plants or secondary infected tissue from plants inoculated with BCTV L2$^-$. CaLCuV is a bipartite geminivirus belonging to the genus *Begomovirus*, while the monopartite BCTV is the type member of the *Curtovirus* genus. H3K9me2 and H3-Ac antibodies were used to assess the association of these marks with viral genomes, with the latter serving as a control for chromatin integrity. Actin and the TEs *Ta3* and *Athila6A* were specificity controls. Precipitated viral DNA was detected by qPCR executed with IR and CP2 primers (Table 1).

ChIP with extracts from primary tissue of BCTV-infected wild-type (Col-0) plants confirmed that H3K9me2 was associated with the IR, although little or no signal was evident at the CP CDS (Fig. 6A). In secondary recovered shoots of wild-type plants infected

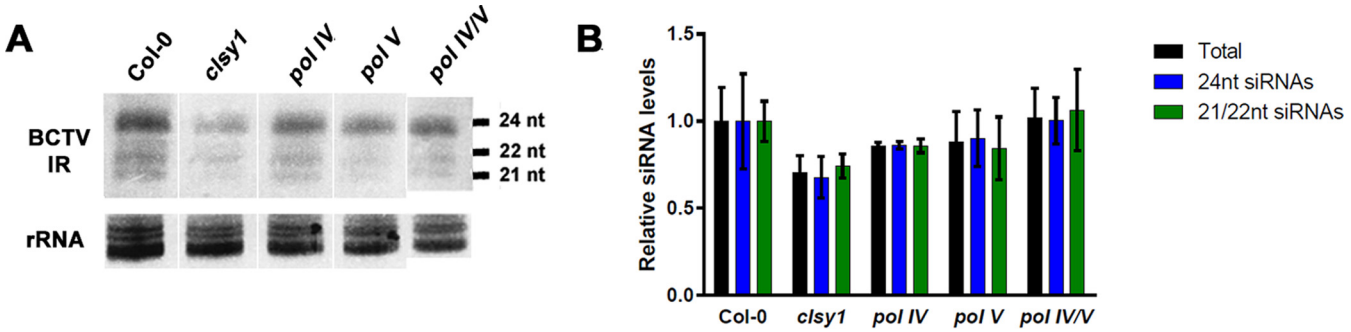

FIG 5 Pol IV and Pol V are not required to generate BCTV-specific siRNAs. (A) Northern blot representative of three independent experiments shows virus-specific small RNAs from BCTV-infected primary tissue of wild-type (Col-0), *clsy1*, *pol IV*, *pol V*, and *pol IV/V* plants. In each case samples consisted of inflorescence tissue pooled from at least three infected plants. The probes used were specific for the BCTV IR. siRNA levels were normalized to rRNA loading controls visualized by ethidium bromide staining. Note that the lanes shown were from the same gel spliced to remove intervening samples. (B) The graph compares relative siRNA levels in wild-type plants and plants harboring the indicated mutations, normalized to rRNA loading controls. Mean levels of total, 24-nt, and 21/22-nt siRNAs observed in wild-type Col-0 plants were set to 1. Data were compiled from three independent experiments. Bars indicate standard errors.

with BCTV L2$^-$, H3K9me2 levels were enriched at the CP CDS compared to primary tissue, correlating with increased cytosine methylation and Pol IV/V occupancy at this locus (Fig. 6B). Remarkably, however, when ChIP was carried out with extracts from infected *pol IV* or *pol V* mutant plants, little or no H3K9me2 was detected at either the IR or the CP CDS in primary (Fig. 6A and C) or secondary infected tissue (Fig. 6B). Similar results were observed with *pol IV/V* double mutant plants (Fig. 6A and B). H3K9me2 was also absent from the CaLCuV IR in infected *pol IV* and *pol V* plants (Fig. 6D). In contrast, abundant H3K9me2 was evident at the endogenous *Ta3* and *Athila6A* TEs in wild-type and mutant plants, confirming that relevant histone methyltransferase activities were not adversely impacted by the polymerase mutations (Fig. 6A to D). ChIP with H3-Ac antibody found this active mark on both the IR and CP CDS in *pol IV*, *pol V*, and *pol IV/V* plants, although it was enriched at the CP CDS and reduced at the IR in mutant compared to wild-type plants (Fig. 6E). Thus, histone H3 continues to associate with viral genomes in the absence of Pol IV and Pol V.

The CaLCuV AL2 protein (also known as AC2 or TrAP) and the related but considerably divergent BCTV L2 interact with adenosine kinase to interfere with the methyl cycle that generates *S*-adenosyl methionine (SAM), an essential methyltransferase cofactor (39, 40, 47). A recent study presented evidence that CaLCuV AL2 also interacts with and inhibits the KYP H3K9 methyltransferase when overexpressed (46). BCTV L2 has yet to be tested for this activity. Nevertheless, H3K9me2 is clearly evident on BCTV and CaLCuV chromatin in wild-type (Col-0) plants (Fig. 6A, C, and D). Further, the lack of H3K9me2 on BCTV L2$^-$ chromatin in *pol IV*, *pol V*, and *pol IV/V* plants makes it unlikely that the viral suppressor is responsible for its absence (Fig. 6B).

Taken together, the results of these experiments clearly indicate that Pol IV and Pol V are individually required for *de novo* deposition of H3K9me2 on geminivirus chromatin but are not needed to maintain previously established H3K9me2 at endogenous TEs.

DISCUSSION

The studies presented here examined the roles of *Arabidopsis* RNA polymerases IV and V in repressive methylation of geminivirus chromatin. Evidence for physical association of Pol IV and Pol V with BCTV chromatin was provided by ChIP experiments, which further demonstrated that these polymerases occupy the promoter-containing viral IR during primary infection and spread to the CP CDS during recovery, correlating with increased cytosine and H3K9 methylation at this locus. While consistent with previously established roles for Pol IV and Pol V in DNA methylation spread (62), why this occurs during recovery, and how Pol IV and Pol V are largely confined to the IR during primary infection, remain unanswered questions.

Genetic studies confirmed Pol IV and Pol V association with viral chromatin and defined individual roles for these polymerases in antiviral defense. Both *pol IV* and *pol V* plants proved hypersusceptible to BCTV, and *pol V* mutants were unable to hypermethylate the viral IR and recover from BCTV L2$^-$ infection. In contrast, the delayed recovery seen in *pol IV* plants, where recovered portions of shoots displayed IR hypermethylation and the initial nonrecovered portions did not, indicated that although Pol IV has an important antiviral role, another activity (or activities) can compensate for its absence. Moreover, the similar delayed recovery observed with *pol IV* and *clsy1* mutants strongly supports the functional association of these enzymes (10).

With the primer set employed in our studies, hypermethylation of the viral IR that occurs during recovery is associated with ~60% cytosine methylation, while only ~40% of cytosines are methylated in nonrecovered *pol IV* and *pol V* shoots. In addition, we previously showed that nonrecovered tissues of *ago4*, *dcl3*, and *drb3* mutants also exhibit this lower, but still substantial, level of IR methylation (36, 54). Thus, the Pol IV-RdDM pathway is essential for IR hypermethylation and host recovery. However, Pol IV-RdDM is clearly not required to establish cytosine methylation on the BCTV genome but rather is necessary for its reinforcement and amplification. Similar conclusions have been reached in several recent studies describing an initiation pathway involving Pol II and RDR6 (RDR6-RdDM) (21–23, 25–27).

While Pol IV and Pol V are not needed to establish cytosine methylation, our ChIP experiments in wild-type and mutant plants demonstrated that Pol IV and Pol V both are required for deposition of H3K9me2 on the chromatin of geminiviruses representing two different genera (BCTV and CaLCuV). Therefore,

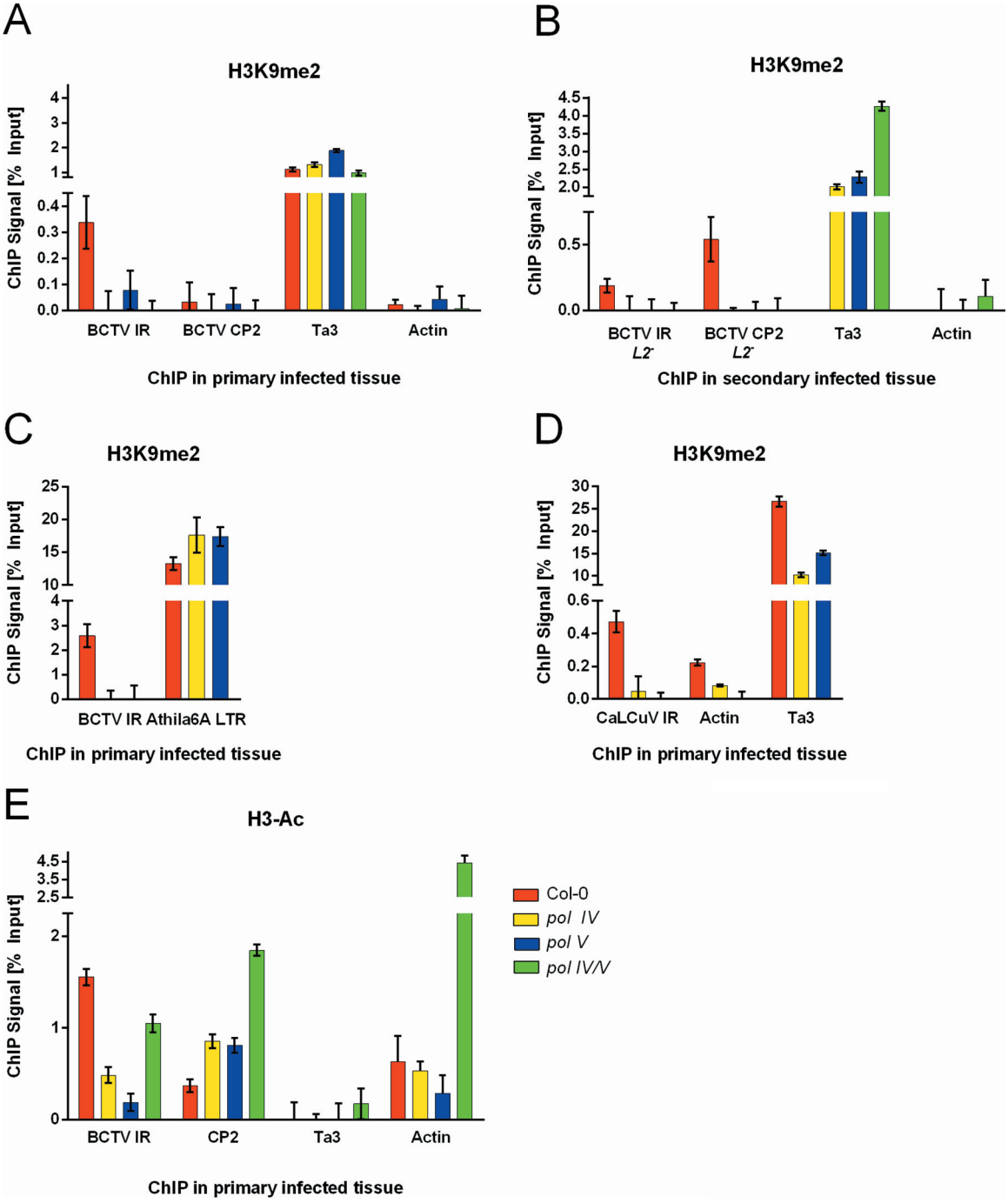

FIG 6 Pol IV and Pol V are required for deposition of H3K9me2 on viral chromatin. Representative ChIP experiments are shown. (A to E) Extracts were prepared from wild-type (Col-0, red bars), *pol IV* (yellow bars), *pol V* (blue bars), or *pol IV/V* (green bars in A, B, and E) plants inoculated with BCTV or CaLCuV (primary infected tissue) or with BCTV L2$^-$ (secondary infected tissue). In all cases, samples consisted of inflorescence tissue pooled from at least three infected plants. ChIP was performed using antibody against H3K9me2 or H3 acetylated at lysine 9 and 14 (H3-Ac) as indicated, and qPCR was carried out in triplicate with primer sets to amplify the viral IR or the CP CDS (CP2) (Table 1). Transposon *Ta3* or *Athila6A* was a positive control for H3K9me2, while actin was a positive control for H3-Ac. The graphs show ChIP signal as a percentage of the input. Graphs in panels A, B, and E are representative of three biological replicates with three technical replicates each (with the exception of BCTV L2$^-$ *pol V*), while panels C and D are representative of two biological replicates with three technical replicates each. The experiment shown in panel C is a partial repeat of panel A but using the Athila6A LTR instead of Ta3 as a positive control. Bars indicate standard errors.

de novo viral DNA methylation can occur in the absence of H3K9 methylation, but cytosine methylation *per se* is not sufficient to initiate *de novo* H3K9me2 methylation. Why the KYP H3K9 methyltransferase, which binds methylated DNA, is apparently unable to access naive viral chromatin is not clear. At this time, we also do not know whether Pol IV-RdDM components other than Pol IV and Pol V are required to establish H3K9 methylation, although it seems likely this pathway is responsible. In any case,

requiring Pol IV and Pol V to both amplify DNA methylation and establish H3K9 methylation may be a strategy to prevent deep silencing of loci that spuriously undergo cytosine methylation due to RDR6-RdDM. Once cytosine methylation is reinforced and H3K9me2 is established, both become independent of Pol IV and Pol V (and RdDM) and can be stabilized by the linked activities of KYP, CMT3, and CMT2, as confirmed by our analysis of *Ta3* and *Athila6A* TEs and numerous other studies (11).

Reductions in virus-derived siRNAs of all size classes observed in mutant plants indicate that Pol IV and Pol V contribute to siRNA biogenesis, although neither is required. However, because Pol IV and Pol V are necessary for host recovery and for the establishment of H3K9me2 on viral chromatin, we argue that their contribution is essential to generate a highly effective antiviral defense. Nevertheless, it is clear that transcription by another polymerase, most likely Pol II, can support the production of viral siRNAs of all size classes, including methylation-associated 24-nt siRNAs. While at odds with studies showing that the bulk of cellular 24-nt siRNAs are Pol IV dependent (57, 58), this likely reflects differences between *de novo* and maintenance processes. It may be that under conditions of active gene expression and prior to extensive methylation of template chromatin, viral dsRNA generated by Pol II-RDR6 is sufficiently abundant to saturate all dicers. Such a mechanism has been proposed to precede transcriptional silencing of an active TE (27).

How Pol IV and Pol V are recruited to geminivirus genomes, and in particular to the promoter-containing IR, remain key unanswered questions. Pol V is recruited to cellular loci by SUVH2 and SUVH9, which bind methylated DNA (17). This is compatible with our results that place viral DNA methylation upstream of H3K9 methylation. On the other hand, Pol IV is recruited to some cellular targets by SHH1, which recognizes H3K9me2 and unmethylated H3K4 (15). Since establishment of H3K9me2 requires Pol IV, it is likely that an SHH1-independent mechanism recruits this polymerase to geminivirus chromatin. Additionally, Pol IV and Pol V might both be recruited by Pol II (see below).

It is curious that the *pol IV/V* double mutation had a lesser impact than single mutants with respect to viral DNA methylation and viral siRNA levels. Why this might be so was not addressed in this study. However, unlike the single mutants, the *pol IV/V* (*nrpd/e2*) mutant lacks the shared second largest subunit and retains the largest catalytic subunits of both polymerases. We speculate that Pol IV and/or Pol V has some residual function in the *nrpd/e2* mutant, perhaps through association with the paralogous second-largest subunit of Pol II. Alternatively, the absence of both Pol IV and Pol V might allow another polymerase (e.g., Pol II) greater access to viral chromatin.

Our observations concerning geminivirus DNA methylation are, for the most part, consistent with a Pol II-RDR6-like mechanism for initiation of cytosine methylation (24). However, an obvious difference is that BCTV DNA methylation does not require Pol V. Thus, we suggest a model borrowed from *Schizosaccharomyces pombe*, where Pol II has both coding and noncoding activities, the latter serving to target H3K9 methylation (63). We propose that Pol II nc transcripts give rise to both siRNAs and scaffold RNAs that direct cytosine methylation machinery primarily to the promoter-containing IR of nascent, unmethylated viral dsDNA replicative forms. Pol II-mediated cytosine methylation could recruit Pol V and possibly Pol IV, or Pol II might directly recruit Pol IV and Pol V and other Pol IV-RdDM components, to reinforce and amplify cytosine methylation and establish H3K9 methylation. In support of our model, *Arabidopsis* Pol II has been shown to interact with Pol IV, Pol V, and AGO4, generate scaffold transcripts, and condition TGS at certain intergenic regions (64). An analysis of the *Arabidopsis* methylome using the same weak *pol II* allele (*nrpb2-3*) employed in the previous study also suggested that Pol II sponsors cytosine methylation at some loci independent of Pol IV and Pol V (65). Our model also provides a possible explanation for the delayed recovery phenotype we observed in *pol IV* and *clsy1* mutants. Cytosine methylation resulting from Pol II nc transcription and/or Pol II-Pol V interaction could recruit Pol V to the viral IR to generate supplementary scaffold transcripts that, in addition to increasing the efficiency of siRNA targeting, could be sliced by AGO4 or AGO6 to generate secondary siRNAs that eventually compensate for the absence of Pol IV (66, 67).

Our studies implicate Pol II in transcription leading to RdDM of geminivirus genomes, and we favor the idea that nc transcription of the IR by Pol II initiates antiviral DNA methylation. Sense and antisense nc transcripts might anneal to create dsRNA, serve as substrates for RDRs, or act as scaffolds, and we have characterized a large number of long nc-RNAs that initiate and/or terminate within the BCTV IR (unpublished results). Because Pol II is normally recruited to promoters, an attractive feature of our model is that it provides a ready explanation for initial targeting of cytosine methylation, as well as Pol IV and Pol V, to the viral IR in primary infected tissue. Alternatively, it has been proposed that divergent readthrough transcription encompassing the entire ~3-kb circular viral genome generates dsRNA that triggers silencing (61). However, there is currently no direct evidence to support this notion, and the abutting 3′ ends of viral transcription units are not hotspots for siRNA production, as might be expected if readthrough were prevalent. It also seems to us that selection would disfavor frequent transcription readthrough events that generate dsRNA.

In conclusion, the studies presented here shed new light on the mechanism of a crucial antiviral defense and highlight the utility of geminiviruses as models for *de novo* methylation of invasive chromatin. We have demonstrated that (i) Pol IV and Pol V are individually involved in antiviral defense and physically associate with BCTV genomes; (ii) Pol IV and Pol V are not required to establish viral DNA methylation but are necessary for its amplification, which leads to host recovery; (iii) Pol IV and Pol V contribute to, but are not required for, biogenesis of virus-derived siRNAs; and (iv) Pol IV and Pol V both are required for *de novo* H3K9 methylation of viral chromatin but not for maintenance of H3K9me2 at endogenous TEs. Current studies are focused on the role of Pol II in antiviral RdDM and the interplay between Pol II, IV, and V.

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